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(71) Applicants: THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY [US/US]; 36th and Spruce Streets, Philadelphia, PA 19104-4268 (US). THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; 133 South 36th Street, Philadelphia, PA 19104-3246 (US).

(72) Inventors: WEINER, David ; 717 Beacon Lane, Merion Station, PA 19151 (US). WILLIAMS, William ; 25 Sycamore Road, Havertown, PA 19083 (US). LEVY, David, N. ; 4046 Chestnut Street, Apt. 3R, Philadelphia, PA 19104 (US).

(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).

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(54) Title: CHIMERIC ENVELOPE PROTEINS FOR VIRAL TARGETING

(57) Abstract

The present invention provides compositions and methods for targeting retroviral vectors specifically to cells of interest for delivery of desired therapeutic or toxic agents. The invention provides a recombinant virus particle comprising (1) a chimeric protein comprising a selected viral envelope gene from which a selected segment has been deleted and into which has been inserted all or an effective portion of a ligand, said deletion rendering the viral particle non-pathogenic and incapable of recombination and said ligand or portion thereof capable of binding to a selected receptor; and (2) an agent for delivery to the target cell selected from the group consisting of a therapeutic agent, a gene or gene product, a diagnostic label, or a toxic agent, which agent is operatively associated with a retroviral packaging sequence.

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CHIMERIC ENVELOPE PROTEINS FOR VIRAL TARGETING

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Field of the Invention

The present invention relates generally to the area of delivery of a biological mediator to a cellular target. More specifically the invention discloses recombinant chimeric molecules comprised of a synthetic nucleic acid sequence encoding a selected ligand capable of targeting binding to a selected cell. This sequence is inserted into a selected viral genome to replace the virus' own targeting signals, thereby rendering the recombinant virus non-pathogenic but capable of infecting a cell bearing the receptor for the ligand.

Background of the Invention

Delivery of a specific biological mediator, e.g., a drug, a toxin, or a gene or gene product, to a specific cellular target in vivo or in vitro requires the very high specificity and efficiency. Viral particles, and in particular retroviruses, continue to be a major focus for use as delivery vehicles for gene therapy or

other biological mediators. The natural host ranges of these viruses has historically determined the cellular subsets which may be targeted by such systems.

The construction of chimeric molecules incorporating binding domains from one source and structural and/or effector domains from another has emerged as an important technique to study protein structure and for the development of novel reagents for the diagnosis and/or treatment of disease.

In the case of retroviruses, the host range is determined by the viral envelope proteins, of which there are two: the transmembrane glycoprotein and the external glycoprotein. The external glycoprotein is anchored to the virus or cell surface via a noncovalent linkage to the transmembrane glycoprotein. Viral binding to the target cell is mediated by the external glycoprotein and this interaction is the major determinant of virus host range within a species.

The envelope proteins of the Human Immunodeficiency Viruses (HIV-1 and HIV-2) confer cell tropism. In nature, the HIV envelope precursor glycoprotein gp160 is cleaved to yield two mature envelope glycoproteins: gp120, the external glycoprotein, and gp41, the transmembrane glycoprotein. The external glycoprotein, gp120, binds the T lymphocyte antigen CD4

and establishes CD4 positive T lymphocytes as a major target for infection in vivo. Other, less well defined, mechanisms also operate in the viral binding of HIV. Bifunctional antibodies incorporating the gp120 binding region of CD4 have allowed the targeting of cytotoxic cells to HIV envelope expressing cells. Soluble CD4 variants incorporating antibody constant regions have shown strong neutralization efficacy in vitro and long half life in vivo.

PCT Patent Application WO91/02805, published March 7, 1991 refers to recombinant retroviruses carrying a vector construct designed to express a selected protein in a target cell. European Patent Application 243,204 refers to the use of retroviral vectors to deliver genes for proteins, such as tumor necrosis factor (TNF).

Construction of a molecule preserving all those attributes necessary for viral infectivity (while perhaps removing those related to cell pathogenesis) will be necessary for the successful development of targeted retroviral vectors. Thus, there remains a need in the art for other compositions and methods for delivering a therapeutic or toxic agent or a diagnostic reagent or label to an infected cell to enable treatment and/or diagnosis of viral and other types of disease.

Summary of the Invention

In one aspect, the present invention provides a chimeric molecule comprising a nucleotide sequence of a selected viral envelope gene from which a selected segment has been deleted and into which has been inserted all or an effective portion of a ligand-encoding nucleotide sequence. The deletion renders the viral sequence non-pathogenic and incapable of recombination into a pathogenic form. The ligand or portion thereof is capable of binding to a selected receptor.

Desirably the viral sequence is that of a retrovirus, although similar constructs may be possible with other viruses, e.g., adenovirus, vaccinia virus and other viruses having similar envelope protein genes. Most desirably the viral sequence is obtained from HIV-1 and/or HIV-2, and the envelope gene in question is gp120 or a portion thereof.

In a preferred embodiment, the chimeric molecule comprises a sequence encoding gp160, wherein the sequence encoding from about amino acids 392 through about 446, which occurs between two cysteine residues, corresponding to the sequence of the HIV-2 ROD isolate has been deleted. Inserted in place of this sequence is the selected ligand-encoding sequence.

The ligand may be selected from among a wide variety of lymphokines, growth factors, hormones, and viral, bacterial or other proteins having their respective receptors present on mammalian or bacterial cells, or viruses. The location of the receptor defines the target cell.

Two particularly preferred ligand-receptor pairs are CD4 and gp120, and Granulocyte-Macrophage Colony Stimulating Factor and its receptor on pluripotent stem cells.

Also present on the chimeric molecule according to this invention is a regulatory control sequence capable of directing the synthesis, replication and expression of the ligand in a selected host cell. Such host cells are preferably mammalian cells, such as COS-7 cells.

In another aspect the present invention includes a vector construct which comprises a chimeric molecule as described above under the regulatory control of a sequence which is capable of directing the synthesis, replication and expression of the chimeric molecule and the ligand in a selected host cell. Also disclosed are cells transfected with the vector of this invention.

In another aspect, the present invention provides a recombinant virus particle comprising an above-described chimeric molecule and an agent for delivery to the target cell selected from the group consisting of a therapeutic agent, a gene or gene product, a diagnostic label, or a toxic agent. The agent is associated with a retroviral packaging sequence. The recombinant virus is preferably characterized by the absence of a complete retroviral env gene. However, the particle does preferably contain retroviral gag and pol genes.

In still a further aspect, the present invention provides a stable cell line, preferably a mammalian cell line, comprising an above-described chimeric molecule and an agent for delivery to the target cell selected from the group consisting of a therapeutic agent, a gene or gene product, a diagnostic label, or a toxic agent, a retroviral packaging sequence, and retroviral gag and pol genes.

In another aspect the invention provides a method for producing a recombinant viral particle of this invention, by culturing under suitable conditions a host cell line which has been transfected with a vector containing the chimeric molecule, a vector containing the therapeutic or toxic agent associated with a retroviral

packaging sequence, and a vector containing retroviral gag and pol genes, but not carrying a complete retroviral env gene.

Still a further aspect of the present invention is a diagnostic composition comprising the chimeric protein produced by expression of the chimeric molecule of this invention.

Further, the invention provides a method for treating a patient for a disease state comprising administering to said patient an effective amount of a recombinant viral particle of the present invention, the administration being either in vivo or ex vivo.

Other aspects and advantages of the present invention are described further in the following detailed description of preferred embodiments of the present invention.

Brief Description of the Drawings

Fig. 1 is an illustration of the sequences involved in the PCR and recombinant construction of embodiments of this invention, as described in Example 2. All primers in this figure are shown 5' to 3'. Note that \TGT\ and \TGC\ indicate codons for Cysteine; and /ACA/ and /GCA indicate the reverse complement of Cysteine.

Fig. 2 is an illustration of the construction of the Env-CD4 chimeric molecules described in detail in Examples 1 and 2, which indicates the PCR reaction and the recombinant reaction producing the chimeric molecule.

Fig. 3 is a drawing of the electrophoretic gel indicating the immunoprecipitation of HIV-2 env-CD4 1 loop.

Fig. 4 is a drawing of the electrophoretic gel indicating the immunoprecipitation of HIV-2 env-CD4 2 loop.

Detailed Description of the Invention

The present invention enables production of a non-pathogenic recombinant viral particle which contains a target host range tailored to a selected cellular subset. This invention enables the expansion or restriction of delivery of biological mediators to only those target cells which would benefit from such treatment.

According to the present invention, the external envelope protein of a selected virus, preferably a retrovirus, may be modified in order to direct virus binding and infection to a particular cellular target cell. Selected cell surface molecules function as novel receptors for infection by a recombinant viral particle

of this invention. Virus infection is thus specifically directed and restricted to cells displaying these antigens via the interaction of the modified viral envelope with the antigens.

The invention encompasses the construction of a chimeric molecule comprising a nucleotide sequence of a selected viral envelope gene from which a selected segment has been deleted and into which has been inserted all or an effective portion of a ligand-encoding nucleotide sequence, said deletion rendering the molecule non-pathogenic and incapable of recombination into a pathogenic virus. The ligand or portion thereof is capable of binding to a selected receptor.

The construction of chimeric molecules of the present invention and use thereof in the construction of non-pathogenic viral particles which are specifically targeted to cell surface proteins may allow the delivery of molecules capable of therapeutic action, including anti-viral agents, with high specificity in vivo or in vitro.

The chimeric molecule of this invention employs nucleotide sequences from the envelope gene of a selected virus. While the concept itself is application to a number of viruses, including vaccinia viruses, adenoviruses, among others, the presently preferred virus

for use in this invention is a retrovirus. Most preferably, the virus selected for the chimeric molecule is Human Immunodeficiency Virus (HIV), either HIV 1 or HIV 2.

The HIV envelope precursor protein gp160 in its uncleaved state retains high affinity for CD4. However, cleavage of gp160 into the external envelope protein gp120 and the transmembrane protein gp41 is necessary for the production of mature envelope capable of mediating fusion with CD4+ cells. The primary sequence requirements and structural requirements for processing of gp160 into mature gp120 and gp41 are not well understood. Deletion and single residue substitution analysis of HIV-1 envelope have determined that cleavage of gp160 into gp120 and gp41 can be disrupted by alterations distal to the cleavage site. Alternatively, nonconservative substitutions near the cleavage site between gp120 and gp41 can have negligible effect on cleavage.

The region of gp120 responsible for the interaction with the target of HIV, e.g., CD4, has been defined by antibody blocking and mutagenesis studies. This CD4 binding site is found near the carboxyl terminus of gp120. The region of gp120 to which most of the CD4 binding function has been attributed is flanked by two

cysteine residues which are disulfide-linked, forming a loop structure called V4. The actual contact region for CD4, or a major part of it, is believed to reside between amino acids 390 and 407 on HIV-1 HXB2 gp120, a strain provided by the National Institutes of Health, and, by analogy, between amino acids 400 and 419 on HIV-2 ROD gp120 (another commonly used NIH strain). The sequences of both strains are publicly available and known to those of skill in the art. The region surrounding and including this putative CD4 binding domain of HIV-2 ROD has structural similarity with immunoglobulin domains. Direct assignment of function for this region of the external envelope protein has been difficult because of the difficulty in reconstituting CD4 binding using small protein analogs derived from region.

To construct an exemplary embodiment of a chimeric molecule of this invention, the portion of gp120 which is considered to be the CD4 binding site is replaced with molecules or portions of molecules capable of targeting selected antigens on selected target cells. The construction of chimeric envelope proteins of this invention employs conventional recombinant DNA technology and various conventional mammalian expression systems Briefly described, coding elements for these chimeric molecules, both the HIV gp120 sequences and the target

ligand sequences are joined into a single DNA molecule containing regulatory sequences capable of directing the synthesis, replication and expression of the chimeric protein in suitable host cells.

Selected target antigens molecules are antigens involved in defined receptor-ligand interactions. A receptor molecule naturally possesses a ligand molecule which binds the receptor molecule with high affinity. The incorporation of the whole ligand molecule, or a part of it which is known to be sufficient for binding to the receptor, into the virus envelope provides a method for redirecting the binding specificity of the viral envelope protein, and hence the whole recombinant virus particle, to cells expressing the receptor.

A non-exclusive list of selected ligand molecules and their receptors includes lymphokines, growth factors, hormones, and viral, bacterial or other proteins. Some specific exemplary ligands are CD4 and Granulocyte-Macrophage Colony Stimulating Factor (GMCSF).

Once constructed by methods described in detail below, a chimeric molecule of this invention is desirably cloned into a selected expression vector containing a regulatory control sequence suitable for directing the replication and expression of the chimeric protein in a selected host cell. Regulatory sequences include

promoter fragments, terminator fragments and other suitable sequences which direct the expression of the protein in an appropriate host cell.

This invention therefore comprises culturing a suitable cell or cell line, which has been transformed with a chimeric molecule coding for expression of a chimeric protein under the control of known regulatory sequences. The vector is transfected by conventional means into a host cell, preferably a mammalian cell for stable expression. This vector may be selected from among many known vectors suitable for mammalian cell expression. Procedures necessary for the construction and use of recombinant vectors and host cells described below are known to those of skill in the art. See, e.g., Sambrook et al, Molecular Cloning. A Laboratory Manual., 2d edition, Cold Spring Harbor Laboratory, New York (1989).

The expressed chimeric protein may be recovered, isolated and purified from the culture medium (or from the cell, if expressed intracellularly) by appropriate means known to one of skill in the art for analysis, such as by syncytia formation assays or immunoprecipitation assays to determine its binding abilities, as described in detail below.

Suitable cells or cell lines for transient or stable expression of the chimeric molecule, and resulting recombinant viral particle containing it are mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and production and purification of the viral particle are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U. S. Patent 4,419,446. Other suitable mammalian cell lines, are the monkey COS-1 cell line, and the CV-1 cell line. Further exemplary mammalian host cells include particularly primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene.

Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

A chimeric envelope protein is then reintroduced into the HIV genome to create an infectious viral clone whose envelope expresses the novel epitope by the following process.

Preferably by a separate transfection, one or more similar vectors containing functional sequences (either the complete sequences or partial sequences encoding enough of these genes to produce their respective functions) encoding the HIV gag and pol genes is introduced into the stable cell line expressing the chimeric protein. These vectors may be constructed by conventional means and such vectors are known and available to those of skill in the art without recourse to undue experimentation. It is likely the chimeric molecule and the gag and pol sequences will become chromosomally integrated into the genome of the same host cell. However, extrachromosomal expression will also be useful.

These sequences are capable of directing the production of a viral particle in the mammalian host cell in operative association with a conventional retrovirus packaging sequence, which may be introduced into the host cell. A retrovirus packaging sequence is a sequence which on an RNA molecule binds to the gag protein when the viral particle forms. Such retrovirus packaging

sequences are known and available to those of skill in the art. However, for use of the retroviral particle to a targeted cell bearing a selected receptor for therapy or diagnosis of a disease state, it is preferred that an agent for delivery to the target cell be selected from the group consisting of a therapeutic agent, a gene or gene product, a diagnostic label, or a toxic agent. Among such agents include toxic compounds useful to kill the target cell, e.g., ricin, or genes or gene product which a cell is lacking, such as in the case of cystic fibrosis, or therapeutic products, such as antibiotics, growth factors, or agents which can bind the receptors of viruses, among others.

Desirably the agent to be delivered to the target cell, e.g., a gene, is cloned by conventional means into another vector. The selected gene is modified to be operatively associated with a retroviral packaging sequence downstream from its transcription initiation site, so that the packaging sequence is within the mRNA. This vector is then introduced into the stable mammalian vector bearing the chimeric protein, and gag and pol genes.

Alternatively, the vectors described above may be co-transfected simultaneously into the host cell. The resulting host cell, upon culture, is selected for

progeny which produce a viral particle which carries the selected gene and the chimeric protein, and the gag and pol genes. Since there is no complete env or gp120 gene in the resulting particle, it is non-pathogenic, and cannot recombine in vivo in a patient, because it lacks essential env sequences.

By virtue of the particle containing a chimeric protein of this invention, it is able to target any cell or virus bearing the appropriate receptor and thus deliver to that cell the desired gene (or other agent).

Although the following description focuses primarily on the construction of a chimeric gp120-CD4 molecule of this invention, it should be understood that the teachings of the invention may permit the construction of a chimeric gp120-other ligand molecule which will perform in an analogous manner. For example, in another embodiment, the receptor-ligand pair Granulocyte-Macrophage Colony Stimulating Factor (GMCSF) and its receptor GMCSF-R receptor (GMCSFr) are employed. GMCSF is central to the development of mature hematopoietic cells of the myeloid lineage. It is expressed on pluripotent stem cells. In the same manner as described above, chimeric HIV envelope proteins are engineered to incorporate the entire mature GMCSF molecule into gp120, thus directing retrovirus packaging cell infection to cells expressing GMCSFr.

Such chimeric molecules may, in an retroviral particle as described above, direct virus binding and infection to cells expressing the receptor of the ligand.

As described below and more specifically in Examples 1 and 2, one exemplary chimeric molecule is constructed wherein the ligand is CD4. The region of CD4 necessary and sufficient for binding HIV envelope has been mapped to a region within the first immunoglobulin-like domain of CD4 using antibody inhibition and mutation analysis. When expressed in eukaryotic cells this region retains high affinity binding to HIV envelope. The CD4 molecule possesses two external disulfide loops, the first loop of which contains the entire gp120 binding domain. Expression of both loops may be necessary in order to preserve high affinity gp120 binding, perhaps as a result of a requirement of both loops for proper folding of the envelope binding domain when produced in some recombinant systems.

In order to construct a chimeric molecule for use in generating a recombinant viral particle to target cells infected with HIV (and which express HIV antigens, including gp120), segments of the CD4 molecule are inserted into gp120 envelope protein in place of the above-described deleted sequence of gp120. One chimeric molecule of the present invention therefore involves

replacement of the V4 region of gp120 with portions of the CD4 molecule sufficient for binding HIV gp120. The novel molecules express epitopes of both proteins. Incorporation of CD4 epitopes into the CD4 binding domain of gp160 has generated chimeric proteins which preserve some functions of native gp160 and eliminate others. The resulting chimeric molecule is a gp120 molecule which is incapable of binding its natural ligand CD4 by virtue of the fact that the CD4 binding domain has been deleted. However, this molecule expresses the CD4 epitope responsible for binding gp120, and thus binds native gp120.

Two methods are used for the design and construction of chimeric molecules. In one example, the chimeric molecule employs the HIV retrovirus as the vector to enable insertion of the targeting sequence into the CD4-binding gene. These embodiments are referred to herein as env-CD4 chimeras.

In one exemplary design a section of gp120 containing the V4 loop as well as additional sequence was deleted. In its place a fragment of CD4 containing either the first disulfide loop or the first and second disulfide loop was inserted.

In a second and preferred method of construction of the chimeric molecule, a cysteine for cysteine replacement was performed between gp120 and CD4. In effect the CD4 binding loops of gp120 are replaced with either the single gp120 binding loop of CD4 or the first two loops of CD4. In this way, as much as possible of the native loop structure of each protein is preserved in the construct. This construct may thus be characterized by the correct folding and function of each loop.

As disclosed below in the following examples, the construction of exemplary chimeric proteins was performed by using a recombinant DNA procedure called gene splicing by Sequence Overlap Extension (SOEing) or, alternatively, Recombinant PCR. Recombinant PCR allows the creation of recombinant DNA molecules of at least 3 kb in length precisely joined regardless of restriction sites present. This has allowed the creation of genes coding for chimeric proteins without any alterations in sequence except where desired.

This technique is used to construct genes encoding chimeric proteins in which the immunoglobulin-like domain containing the CD4 binding domain of HIV-2 gp120 is replaced by regions of CD4 responsible for binding HIV gp120. Chimeric HIV-2 Env-CD4 molecules were

created whose junctions are the cysteine residues that define the subunit structure of the native proteins. These cysteine residues are disulfide bonded in the native proteins and domain exchange is performed at these points in the anticipation that cysteine binding patterns might be preserved in the chimeric proteins.

Two such chimeric envelope proteins are described in the following examples: one (ROD Env-1 loop) contains the first cysteine loop of CD4 and the other (ROD Env-2 loop) contains the first two loops of the CD4 molecule in place of the CD4 binding domain of HIV-2 ROD gp120.

These molecules have been expressed in COS cells in a transient assay. These chimeric molecules do not appear to be processed into mature proteins and do not mediate fusion between transfected cells expressing them and CD4+ cells. As shown in Example 4, anti-CD4 antibodies fail to precipitate the envelope-CD4 chimera containing the first loop of CD4. Both the region of gp120 replaced and the region of CD4 inserted are of similar size, are involved in receptor-ligand interactions (and thus are exposed on the surface of their native molecules) and are structurally related (insofar as they are members of the immunoglobulin superfamily). It is expected that the CD4 epitope would

be preserved in the context of the HIV-2 envelope. The chimeric protein, ROD Env-1 loop, appears to dimerize, as has been described for native HIV-2 gp160.

Surprisingly, however, incorporation of a substantially larger segment of the CD4 molecule is required for the preservation of epitopes necessary for antibody binding. The two loop chimera expresses epitopes of CD4 which are capable of binding native HIV envelope protein, as the chimeric envelope is precipitable by anti-CD4 antibodies in the immunoprecipitation assay described below.

As noted above, expression of both the first two immunoglobulin-like domains of CD4 has previously been observed in some cases to be necessary to retain proper folding. This requirement is confirmed when the HIV envelope binding domain of CD4 is expressed in the context of another molecule such as HIV envelope.

It is contemplated that the expression efficiency of the chimeric proteins may be a necessary prerequisite for successful analysis and detection of surface expression of the chimeric proteins.

These chimeric molecules of this invention have significant use in therapeutic and possibly diagnostic application. Where the ligand is CD4, the viral particle will bind to gp120, and thereby primarily cells infected

with HIV. Thus, this embodiment of the technology will have utility in the treatment of HIV infection, as it allows the specific delivery to infected cells of biological mediators which can inhibit HIV spread.

In an analogous manner, another selected ligand sequence may be incorporated into the deleted portion of a gp120 gene sequence, and a chimeric molecule capable of targeting to a cell or virus bearing the ligand's receptor is generated. Where the ligand is, e.g., GMCSF, the viral particle will bind to stem cells. This embodiment permits the delivery of effectors (such as genes) to the pluripotent stem cells to correct blood-born disorders of metabolism or to "intracellularly immunize" lineages of leukocytes against infectious agents such as viruses.

Other ligands in chimeric molecules constructed according to this invention will be useful in other therapies.

The following examples are illustrative in nature and the disclosure is not limited thereto.

Example 1: Gene Construction

Native HIV-2 envelope gene, gp160-rev, was amplified by the PCR technique [see, e.g., H. A. Erlich, ed., "PCR Technology", Stockton Press: New York (1989)

and H. A. Erlich et al, (eds), "Polymerase Chain Reaction" in Curr. Comm. Mol. Biol., Cold Spring Harbor Laboratory Press, New York (1989)] from an HIV isolate, HIV-2 ROD, cloned in phage lambda [National Institutes of Health] as follows. The complete molecular clone is referred to as HIV-2 ROD-lambda.

The PCR primers, A and H from Fig. 1, were designed to amplify the region of the HIV-2 genome encompassing the rev and env open reading frames and relevant splice sites. The design of these primers was based on the known published sequence of the HIV-2 ROD virus.

They amplified a segment of 2708 base pairs from HIV-2 ROD-lambda, as illustrated in the top third of Fig. 2. The resulting PCR products were cloned into the plasmid pCDNA I/NEO [commercially available from InVitrogen], a vector allowing high expression in a mammalian cell transient assay system (COS), using XhoI (5') and XbaI (3') restriction sequences incorporated into the primers as shown in Fig. 1.

Example 2: Construction of Chimeric env-CD4 DNA Molecules by Recombinant PCR

Primers were designed to amplify three DNA fragments to be joined together to construct the final molecule.

HIV-2 rev-160 primers of Fig. 1 were used in the PCR technique conducted on the previously amplified 2708 base pair segment of the HIV-2 rev-envelope gene amplified as described above in a manner illustrated in the graph of Fig. 2. As illustrated in the top third portion of Fig. 2, primers A and B were used to amplify a fragment of the env gene, corresponding to the first exon of rev and amino acids 1-392 of the HIV-2 gp160; primers G, F and H were used to amplify another portion of the rev gene corresponding to amino acids 446-858 of HIV-2 gp160, the second exon of rev. The primers A, B, G, F, and H of Fig. 1 incorporated approximately 15 base pairs homologous to the CD4 region to be joined so that the resulting PCR products would be readily fused to the CD4 loop sequences. The resulting PCR products of these priming events are illustrated in Fig. 2 as two portions of the env and rev genes respectively, each containing a cross-hatched sticky end for the CD4 loop sequence.

Amplification of these segments omitted the coding sequence for 54 amino acids of gp160 corresponding to the region between the two cysteines at amino acid numbers 392 and 446.

Still referring to Fig. 2, in the middle third of the figure, another priming event was performed using the published CD4 template 4pMV7 and primers C, D, and E

from Fig. 1. These primers were used to amplify the regions of CD4 to be incorporated into the gp160 molecule. These amplified segments encoded either the first cysteine loop of CD4 (amino acid numbers 16-84 from the published sequence) or the first two loops of CD4 (amino acid numbers 16-159 from the published sequence).

Finally, the bottom third of Fig. 2 depicts the recombination event between these three primary PCR products, i.e., the env and rev sequences with the appropriate CD4 loop structure. To recombine the three primary PCR products, a PCR reaction was performed in which the products to be joined were mixed together with the far 3' and 5' envelope primers A and H of Fig. 1. Recombination between the three primary PCR products occurred by virtue of the homologous regions incorporated in the primary PCR reactions and the recombination product was amplified by the external primers. This final product is illustrated by the last bar graph on Fig. 2. All products were confirmed to be correct recombinants by restriction analysis.

The two chimeric Env-CD4 molecules created (ROD Env-1 loop and ROD Env-2 loop) retain many characteristics of native HIV-2 gp160, but to varying degrees.

The amplified recombinant sequences were subcloned into the XhoI-XbaI sites of pCDNA I/NEO to provide suitable plasmids for transfection.

Example 3: Transient Transfection Assay

For transfection, COS-7 (SV40 transformed monkey kidney) cells were grown in DMEM supplemented with 10% fetal calf serum and penicillin and streptomycin. For each assay below, approximately $1X10^6$ COS-7 cells were transfected with approximately 2μg of double cesium banded DNA by DEAE dextran transfection in the presence of chloroquine. Cells were harvested and/or assays performed at 40-72 hours post transfection. Transfectants were analyzed by immunoprecipitation and by a cell fusion assay described below.

Recombinant protein expression in this system yields HIV-2 envelope protein with the same biological characteristics as observed in virally infected cells.

Example 4: Syncytium Assay

To determine whether the amplified env gene of HIV-2 ROD could mediate the fusion of HIV-2 infected cells with uninfected cells, the following assay was conducted. For this assay, Sup-T1, a CD4+ human non-Hodgkins T lymphoma [American Type Culture Collection,

Rockville, MD] was grown in RPMI medium with 10% fetal calf serum (FCS) and penicillin/streptomycin.

The transfected cells of Example 1 were incubated with Sup-T1 cells at day 1 following transfection and examined visually for the formation of multinucleated giant cells (syncytia) at regular intervals until COS cell death caused by the overreplication of plasmid DNA.

The kinetics of fusion with CD4+ cells is faster for HIV-2 envelope-expressing cells than for HIV-1 HXB2 expressing cells (a strain cloned and expressed in a similar manner) [NIH]. This phenomenon reiterates the difference in fusion kinetics observed for infected cells. This assay demonstrated that the biological function of these cloned HIV envelope segments is preserved. This assay may also be employed to demonstrate that the biological function of the env portion of the recombinant molecule is also retained.

Example 5: Immunoprecipitations

The recombinant molecules described above are tested in an immunoprecipitation assay with antibodies to the native HIV env and CD4 proteins, to determine if the segments in the recombinant molecules resemble the native protein, as shown by antibody recognition. For example,

the recognition of the HIV env is essential so that the recombinant molecule retains characteristics necessary for infectivity of target cells. The recognition of the CD4 loop(s) is necessary to determine if the segment of this ligand retains its ability to bind to its receptor.

A. HIV-2 env-CD4 1 loop.

After about 48 hours, approximately $1x10^6$ COS-7 cells transfected with the recombinant molecule containing one CD4 loop and described in Example 3 were metabolically labelled with ^{35}S-methionine and -cysteine for 4 to 16 hours in a 5% CO_2 atmosphere. Cells were solubilized and lysed in standard lysis buffer containing 0.5% Triton-X 100 and 0.5% Deoxycholate.

HIV-2 envelope proteins were immunoprecipitated using pig anti-HIV-2 serum [Verigen Corp.], normal pig serum (negative control), a cocktail of anti-CD4 antibodies or an irrelevant monoclonal antibody, bound to protein A agarose. Anti-HIV-2 antibodies were either immune patient serum [NIH AIDS Research Reagent Reference Program (ARRRP)] or the pig serum against HIV-2. Anti-CD4 antibodies used to precipitate chimeric proteins were a mixture of Leu-3a, SIM.2 [ARRRP], SIM.4 [ARRRP], and a R-anti-CD4 polyclonal serum [ARRRP].

Labelled proteins were reduced and then run on 10% SDS-PAGE and autoradiographed. The resulting gel is illustrated in Fig. 3. The chimeric molecule containing only one CD4 loop is not precipitable by anti-CD4 antibodies, indicating that the CD4 epitopes are improperly expressed and/or hidden. The ROD Env-1 loop chimeric protein is produced in COS cells as a protein of approximate molecular weight of 98 kd, the predicted molecular weight based on the size and glycosylation patterns of the native subunits. The ROD Env-1 loop chimera oligimerizes to yield a band at approximately 300 kd in the same manner as native HIV-2 gp160. The region(s) of the envelope protein responsible for gp160 oligomerization are not known but the region deleted (a.a. 393-445) does not appear to be required for oligomerization. However, the single loop chimera appears to be cleaved poorly if at all into the transmembrane and external proteins.

B. HIV-2 env-CD4 2 loop.

As described above for part A, approximately $1x10^6$ COS-7 cells transfected with 2 μg plasmid DNA containing two loops of CD4 using DEAE-dextran in the presence of chloroquine were, after 48 hours, metabolically labelled with ^{35}S-methionine and -

cysteine for between 4 to 16 hours then solubilized in the above-described lysis buffer. Immunoprecipitation was performed using human anti-HIV-2 serum or the cocktail of anti-CD4 antibodies bound to protein A agarose, described above.

Labelled proteins were reduced, and then run on 10% SDS-PAGE and autoradiographed. The resulting gel is illustrated in Fig. 4. The chimeric protein ROD Env-2 loop is precipitable with anti-CD4 antibodies to reveal a single band at approximate molecular weight 160 kd, the size predicted for an uncleaved chimeric protein. No oligimization has been observed for the ROD Env-2 loop protein. However, proteins of such high molecular weight are poorly loaded and resolved (note the barely loaded oligimer bands for native gp160) in these gels and definitive analysis of this question awaits further study.

In addition, the yield of ROD Env-2 loop is substantially lower than for native envelope or for ROD Env-1 loop. Substitution of heterologous leader sequences has been observed to be necessary for high expression of HIV envelopes and mutant proteins in several systems and may be of benefit in this system.

Example 6: Construction of a Chimeric Molecule with a GMCSF Ligand

In the construction of an HIV env-GMCSF chimeric protein, a section of gp120 that is greater than just the V4 loop is replaced in order to (1) insert the entire mature GMCSF molecule and (2) preserve much as possible the original size of gp120. Studies have shown that most or all of the GMCSF molecule is required for binding to its receptor. As there is no cysteine for cysteine exchange possible between GMCSF and gp120, preservation of the relative size of the envelope molecule is desired. Additional constructs of env-GMCSF chimeras may also preserve of as much of gp120 as possible.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. For example, use of other appropriate receptor-ligand systems than CD4-gp120 can be employed and selected retroviruses other than HIV-1 and HIV-2 are contemplated in the performance of this invention. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A chimeric molecule comprising a nucleotide sequence of a selected viral envelope gene from which a selected segment has been deleted and into which has been inserted all or an effective portion of a ligand-encoding nucleotide sequence, said deletion rendering the viral non-pathogenic and incapable of recombination and said ligand or portion thereof capable of binding to a selected receptor.

2. The molecule according to claim 1 wherein said virus is a retrovirus.

3. The molecule according to claim 2 wherein said virus is HIV-1.

4. The molecule according to claim 2 wherein said virus is HIV-2.

5. The molecule according to claim 4 wherein said viral envelope gene is gp160.

6. The molecule according to claim 5 wherein said deletion is from the sequence encoding from about amino acids 392 through about 446 of gp160, which occurs between two cysteine residues, corresponding to the sequence of the HIV-2 ROD isolate.

7. The molecule according to claim 1 wherein said ligand is CD4 and said receptor is gp120.

8. The molecule according to claim 1 wherein said ligand is GMCSF and said receptor is the GMCSF receptor on pluripotent stem cells.

9. The molecule according to claim 1 wherein said ligand is selected from other lymphokines, growth factors, hormones, and viral, bacterial or other proteins and the receptor is selected from receptors of lymphokines, growth factors, hormones and cell-surface proteins and viral and bacterial proteins.

10. The molecule according to claim 1 further comprising a regulatory control sequence capable of directing the synthesis, replication and expression of the ligand.

11. A recombinant virus particle comprising (1) a chimeric protein comprising a selected viral envelope gene from which a selected segment has been deleted and into which has been inserted all or an effective portion of a ligand, said deletion rendering the viral particle non-pathogenic and incapable of recombination and said ligand or portion thereof capable of binding to a selected receptor; and (2) an agent for delivery to the target cell selected from the group consisting of a therapeutic agent, a gene or gene product, a diagnostic label, or a toxic agent, which agent is operatively associated with a retroviral packaging sequence.

12. The recombinant virus particle according to claim 11 characterized by the absence of a complete retroviral env gene.

13. A method for treating a patient for disease comprising administering to said patient an effective amount of a recombinant viral particle of claim 11.

14. A vector construct which comprises a chimeric molecule comprising a nucleotide sequence of a selected viral envelope gene from which a selected segment has been deleted and into which has been inserted all or an effective portion of a ligand-encoding nucleotide sequence, said deletion rendering the virus non-pathogenic and incapable of recombination and said ligand or portion thereof capable of binding to a selected receptor, said chimeric molecule under the regulatory control of a sequence which is capable of directing the synthesis, replication and expression of the chimeric protein and the ligand in a selected host cell.

15. A cell transfected with the vector of claim 14.

16. A chimeric protein produced by the expression of the molecule of claim 1 in a suitable cell.

17. A stable cell line comprising a chimeric molecule comprising a nucleotide sequence of a selected viral envelope gene from which a selected segment has been deleted and into which has been inserted all or an effective portion of a ligand-encoding nucleotide sequence, said deletion rendering the virus non-pathogenic and incapable of recombination and said ligand or portion thereof capable of binding to a selected receptor and an agent for delivery to the target cell selected from the group consisting of a therapeutic agent, a gene or gene product, a diagnostic label, or a toxic agent, said agent associated with a retroviral packaging sequence, and retroviral gag and pol genes.

FIG. 1

PRIMERS FOR ROD ENV-CD4 CHIMERAS

A.	GGCTGCctcgagCAAGGGGCTCGGGATATG	5' HIV-2 ROD XhoI, REV ATG
B.	CD4 env CTTCTGGGAAGCTGT/GCA/GTTAGTCCACATGTA	3' HIV-2 ROD WITH CD4 TAG
C.	/TGT/ACAGCTTCCCAGAAG	5' CD4 FIRST CYSTEINE
D.	/ACA/GATGTAAGTATC	3' CD4 SECOND CYSTEINE - 1 LOOP
E.	/GCA/TGTCCAGGTGCC	3' CD4 FOURTH CYSTEINE - 2 LOOPS
F.	CD4 env GATACTTACATC/TGT/AACTCAACAGTAACC	5' HIV-2 ROD WITH CD4 TAG - 1 LOOP
G.	CD4 env GGCACCTGGACA/TGC/AACTCAACAGTAAC	5' HIV-2 ROD WITH CD4 TAG - 2 LOOPS
H.	GCTAGCtctagaACTGCCGTCCCTCACAG GAGGCGATTTC	3' HIV-2 ROD XbaI, ENV STOP

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FIG. 2 ENV-CD4 CONSTRUCTION - RECOMBINANT PCR

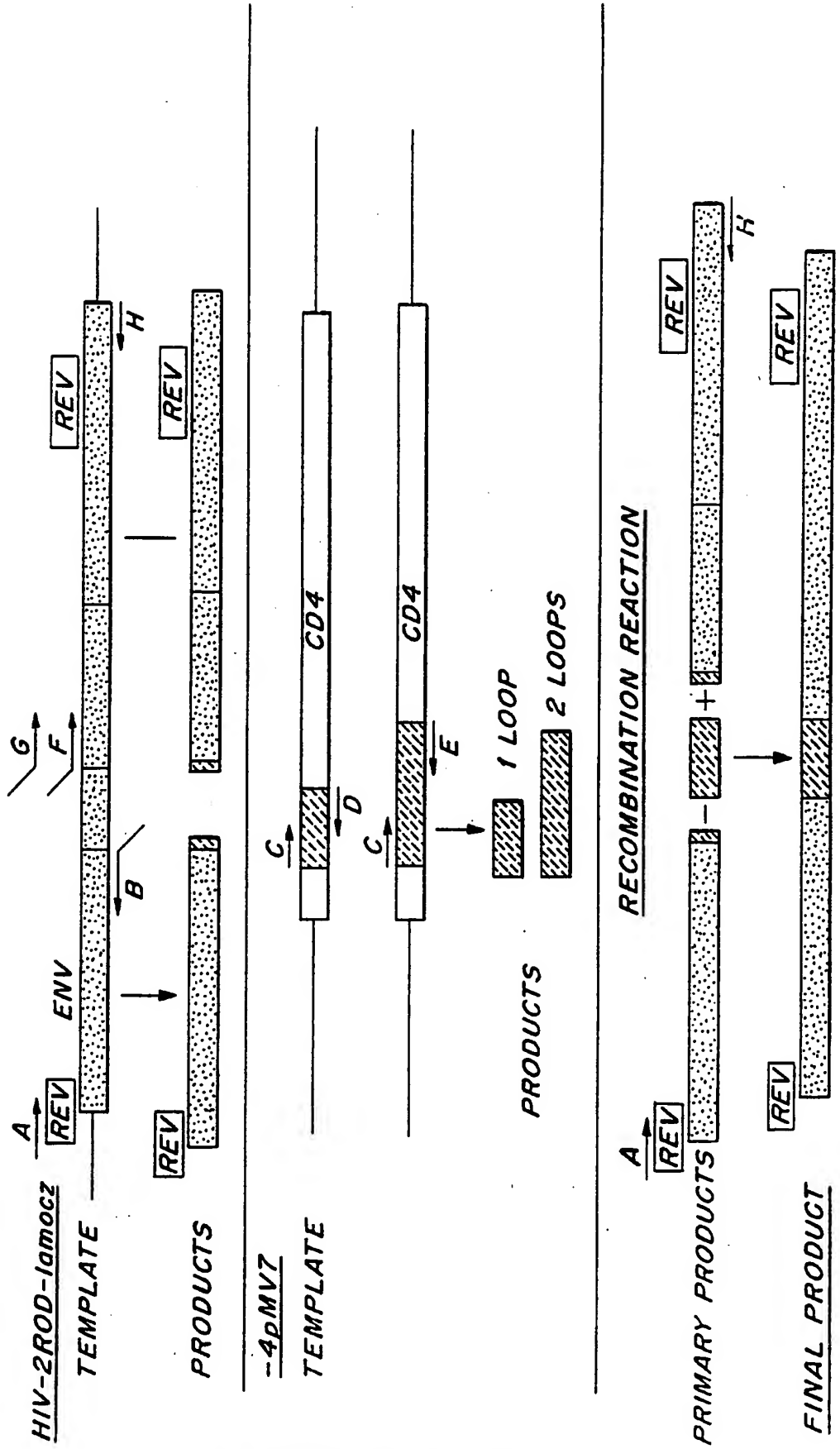


FIG. 3

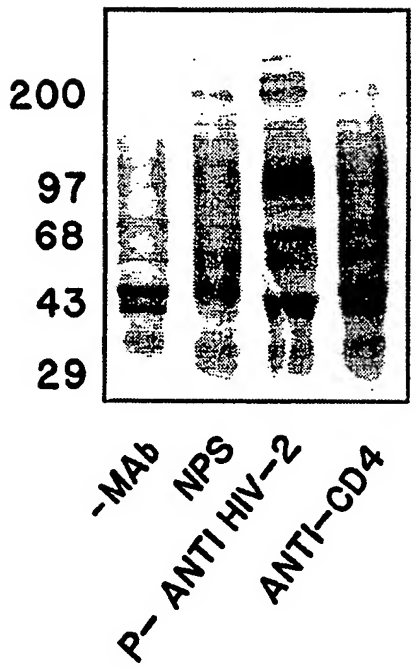
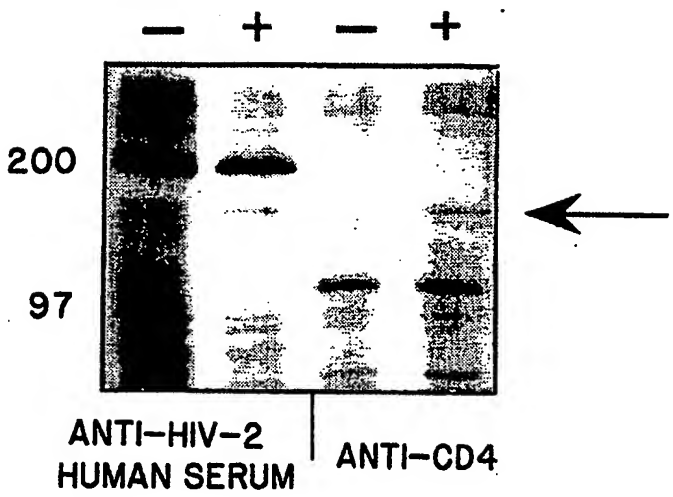


FIG. 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05159

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 35/76; C07H 21/04; C12N 5/10, 7/01, 15/86; C12P 21/00
US CL :424/93R; 435/236, 240.1, 240.2, 320.1; 530/350; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93R; 435/236, 240.1, 240.2, 320.1; 530/350; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS PREVIEWS 1985+, MEDLINE 1975+ NTIS, CA SEARCH, BIOTECHNOLOGY ABSTACTS 1982+, AIDSLINE, WORLD PATENT INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Science, Volume 250, issued 07 December 1990, J. A. T. Young et al, "Efficient Incorporation of Human CD4 Protein into Avian Leukosis Virus Particles," pages 1421-1423. See entire article.	1,2,9,10,14-16 1-17
Y	WO,A, 90/12087 (Henderson et al) 18 October 1990. See page 18, line 21 through page 19, line 10; also claim 3 and 10.	8,17
Y	WO,A, 89/09271 (Gruber et al) 05 October 1989. See page 18, lines 1-22	8,17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 September 1992	Date of mailing of the international search report 15 SEP 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JOHNNY F. RAILEY II Telephone No. (703) 308-0281

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